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## Block of Kcnk3 by Protons

EVIDENCE THAT 2-P-DOMAIN POTASSIUM CHANNEL SUBUNITS FUNCTION AS HOMODIMERS\*

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KCNK subunits have two pore-forming P domains and four predicted transmembrane segments. To assess the number of subunits in each pore, we studied external proton block of Kcnk3, a subunit prominent in rodent heart and brain. Consistent with a pore-blocking mechanism, inhibition was dependent on voltage, potassium concentration, and a histidine in the first P domain (P1H). Thus, at pH 6.8 with 20 mM potassium half the current passed by P1H channels was blocked (apparently via two sites ~10% into the electrical field) whereas channels with an asparagine substitution (P1N) were fully active. Furthermore, pore blockade by barium was sensitive to pH in P1H but not P1N channels. Although linking two Kcnk3 subunits in tandem to produce P1H-P1H and P1N-P1N channels bearing four P domains did not alter these attributes, the mixed tandems P1H-P1N and P1N-P1H were half-blocked at pH ~6.4, apparently via a single site. This implicates a dimeric structure for Kcnk3 channels with two (and only two) P1 domains in each pore and argues that P2 domains also contribute to pore formation.

Leak currents are key to normal electrical activity of sympathetic ganglia, myelinated axons, carotid body type 1 cells, and cardiac myocytes (1). 50 years after their description (2, 3) leak currents have been revealed to pass via potassium channels formed by subunits with two P domains (1). Preceded by TOK1, a non-voltage-gated outward rectifier from *Saccharomyces cerevisiae* with eight predicted membrane-spanning segments (a 2P/8TM<sup>1</sup> topology) (4), the first canonical leak channel, KCNK0, was isolated from *Drosophila melanogaster* and has a predicted 2P/4TM topology (5–7). To date, 15 mammalian genes (*KCNK1–15*) have been identified to encode subunits with a 2P/4TM topology (1). Family members that show function (*KCNK0*, -2, -3, -4, -5, -6, -9, -10, -13, and -15) are potassium-selective leak channels; open at rest, they pass potassium

without apparent delay in response to changes in membrane potential across the physiological voltage range. The functional repertoire of KCNK channels is, however, more varied, as phosphorylation can reversibly transform cloned and hippocampal KCNK2 between an open and voltage-dependent phenotype (8). Other KCNK subunits have yet to reveal their biophysical attributes (*KCNK1*, -7/8, and -12).

Previously, we studied the genomic structure, cardiac localization, and biophysical properties of murine Kcnk3 (9). Thus, at steady-state, Kcnk3 channels behave like potassium-selective, transmembrane holes inhibited by physiological levels of protons. With voltage steps, Kcnk3 currents activate (or decay) in two phases; one phase appears to be instantaneous, and another appears to be time-dependent ( $\tau \sim 5$  ms). Both proton block and time-dependent gating are potassium-sensitive, producing an anomalous increase in outward flux as external potassium levels rise because of decreased proton block. At physiological levels of potassium, proton inhibition at -120 mV is half-maximal at ~pH 7.6, indicating that Kcnk3 is poised to sense and respond to changes in pH value. Moreover, Kcnk3-like channels have been recorded in rat central nervous system nuclei, where they were shown to influence spike frequency in response to volatile anesthetics and neurotransmitters (10–14), and in murine cardiac myocytes, where they appear to contribute to the action potential plateau current called IKp (9, 15). These findings suggest a potential role for Kcnk3 in the physiology of cerebral and cardiac acidosis.

Four KCNK channels are notably sensitive to extracellular pH in the physiological range, (*KCNK3*, -5, -9, and -15) (1, 16). *KCNK3* and -9 appear to be closely related sharing 54% amino acid identity and a histidine at position 98 in the first P domain (P1) in the midst of signature sequence residues that form the potassium selectivity filter (GYGH); *KCNK5* and -15 do not possess this histidine. Kim *et al.* (17) have demonstrated that histidine 98 in *KCNK9* confers sensitivity to external pH. In this study, we demonstrate a similar role for histidine 98 in Kcnk3 and employ this finding to evaluate subunit stoichiometry. Our findings support the conclusions that there are two P1 domains present in the channel that contribute equivalently to pore formation and, thus, that two Kcnk3 subunits assemble to form each channel.

## MATERIALS AND METHODS

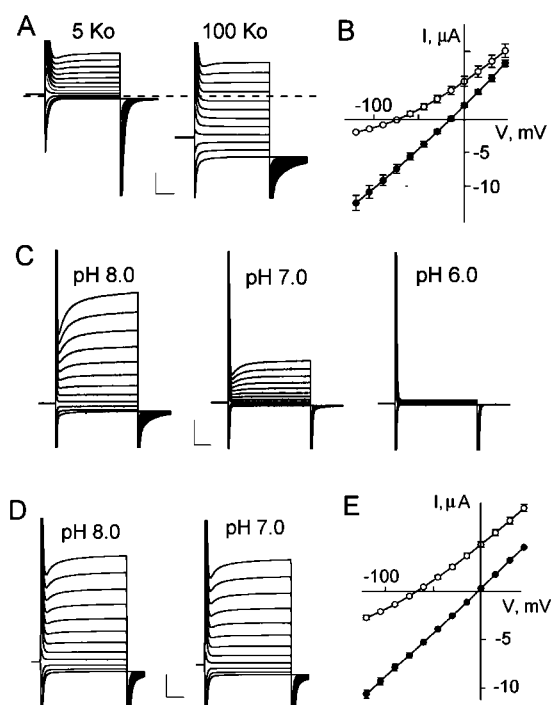
**Molecular Biology**—Cloning of *Kcnk3* (GenBank<sup>TM</sup> accession number AB008537) and the pBF2 vector have been described (9). Point mutants were produced by *Pfu* mutagenesis with a Quickchange<sup>TM</sup> kit (Stratagene, Inc., La Jolla, CA) followed by insertion of mutant gene fragments into translationally silent restriction sites. Sequences were confirmed by DNA sequencing. To produce dimer constructs, a cDNA fragment encoding all but the first 6 amino acids of Kcnk3 was excised (with *FspI* and *EcoRI*) and ligated behind another copy of the gene replacing the last 71 amino acids (from *ScaI* to *EcoRI*). The result was two linked but truncated Kcnk3 subunits: the first lacks 71 C-terminal residues, and the second lacks 6 N-terminal residues. Wild type (P1H) and H98N (P1N) subunits were combined in this fashion to form P1H-P1H, P1H-P1N, P1N-P1H, and P1N-P1N. cRNA was produced with T3 RNA polymerase and a kit (Ambion, Austin, TX).

**Electrophysiology**—Oocytes were isolated from *Xenopus laevis* frogs (Nasco, Atkinson, WI), treated with collagenase to ease follicle removal, and injected with 0.1–0.4 ng of Kcnk3 cRNA in 46 nl of sterile water. Currents were measured 1–4 days after injection by two-electrode voltage clamp using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA). Data were sampled at 4 kHz, filtered at 1 kHz, and analyzed using Pulse (Instrutech, Great Neck, NY) and Sigmaplot

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<sup>1</sup> The abbreviations used are: TM, transmembrane; MES, 4-morpholineethanesulfonic acid.



**FIG. 1. Kcnk3 channels are open rectifiers, show time-dependent gating, and are blocked by lowered pH values in wild type but not mutant form.** *A*, Kcnk3 currents in whole-cell mode for a representative oocyte bathed in 5 and then 100 mM potassium (*Ko*) held at  $-80$  mV and pulsed from  $-120$  to  $45$  mV in  $15$ -mV voltage steps lasting  $50$  ms, followed by a  $20$ -ms step to  $-120$  mV with a  $1$ -s interpulse interval. The dashed line indicates zero current level. The time-dependent phase of current development is partially obscured in whole cell mode by the capacitance transient. Scale bars,  $2$   $\mu$ A,  $20$  ms. *B*, steady-state current-voltage relationships for groups of five oocytes studied as in panel *A* in  $5$  (open circles) and then  $100$  mM potassium (filled circles); mean  $\pm$  S.E. *C*, inhibition of Kcnk3 channels by lowered external pH values. Kcnk3 currents in whole-cell mode for a representative oocyte bathed in  $5$  mM potassium at pH  $8.0$ ,  $7.0$ , and  $6.0$ ; protocol was as in *A*. *D*, P1N channels show little inhibition by lowered external pH. Whole-cell mode for an oocyte bathed in  $5$  mM potassium at pH  $8.0$  and  $7.0$ ; protocol was as in *A*. *E*, steady-state current-voltage relationships for six oocytes as in panel *A* in  $5$  mM (open circles) and then  $100$  mM (filled circles) potassium solution; mean  $\pm$  S.E.

(Jandel Scientific, San Rafael, CA) software. Electrodes of  $1.5$ -mm borosilicate glass tubes (Garner Glass Co., Claremont, CA) contained  $3$  M KCl and had resistances of  $0.3$  to  $1$  megohms. Oocytes were studied at room temperature with perfusion at  $0.4$ – $1$  ml/min using  $5$  mM potassium solution as follows (in mM):  $93$  NaCl,  $5$  KCl,  $1$  MgCl<sub>2</sub>,  $0.3$  CaCl<sub>2</sub>,  $5$  HEPES, pH  $7.4$ , with NaOH. As indicated, KCl was isotonicly substituted for NaCl. At pH  $6.0$ , MES was substituted for HEPES.

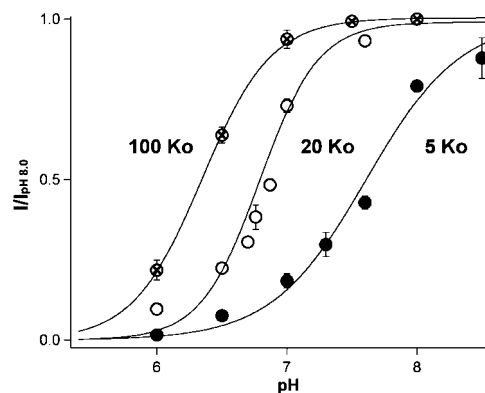
**Calculations**—Dose response curves were fit to Equation 1,

$$\left[ 1 + \left( \frac{[B]}{K_{1/2}} \right)^n \right]^{-1} \quad (\text{Eq. 1})$$

where  $[B]$  is proton or barium concentration,  $K_{1/2}$  is the concentration of  $B$  required to achieve  $50\%$  inhibition, and  $n$  is the Hill coefficient. Voltage dependence was modeled for data accumulated in  $20$  mM potassium solution using a simplification of the Woodhull approach (18), represented by Equation 2,

$$\frac{I_{\max}}{I} = 1 + \left( \frac{[B]}{K_{1/2}} \exp \left[ \frac{-z\delta FV}{RT} \right] \right)^n \quad (\text{Eq. 2})$$

where  $[B]$  is proton or barium concentration,  $n$  is the Hill coefficient,  $z$  is the electronic charge of the blocker, and  $\delta$  is the apparent electrical distance traversed by the blocker to reach its site.



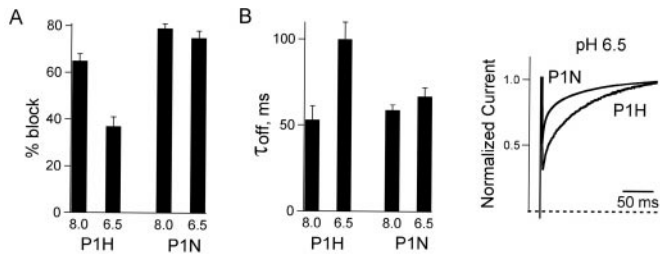
**FIG. 2. Kcnk3 channels are inhibited by protons in an external potassium-dependent manner.** The dependence of Kcnk3 current at  $-120$  mV on bath pH (mean  $\pm$  S.E.) for groups of  $10$ – $20$  oocytes in  $5$ ,  $20$ , and  $100$  mM potassium solution. The solid lines represent fits of the data in Equation 1.  $K_{1/2}$  was determined to be  $7.6 \pm 0.6$ ,  $6.83 \pm 0.01$ , and  $6.35 \pm 0.02$ , and Hill coefficients were  $0.96 \pm 0.06$ ,  $2.02 \pm 0.14$ , and  $1.86 \pm 0.21$  for  $5$ ,  $20$ , and  $100$  mM potassium (*Ko*), respectively.

## RESULTS

**Native Kcnk3 and a Mutant**—Native Kcnk3 channels expressed in oocytes are notable for at least four reasons. First, the currents activate and deactivate in response to voltage changes in two phases, one instantaneous, another time-dependent (Fig. 1*A*). A non-zero holding current at voltages from  $-120$  to  $60$  mV (Fig. 1, *A* and *B*) indicates that the channels are open across the physiological voltage range, as has been observed directly at the single channel level (9). Second, Kcnk3 currents are non-inactivating, remaining at a stable level until voltage is again altered. Third, Kcnk3 currents show open rectification; as expected for an open, potassium-selective portal, the relationship of current and voltage becomes linear as potassium is raised to nearly equal levels across the membrane (Fig. 1*B*). Conversely, at physiological levels of bath potassium ( $\sim 5$  mM), Kcnk3 passes large outward currents and small inward currents, because permeant ions flow more readily from a side of high concentration. Fourth, Kcnk3 channels are inhibited by protons in the physiological pH range. Currents are close to maximal at pH  $8.0$ , partially suppressed at pH  $7.0$ , and essentially absent at pH  $6.0$  (Fig. 1*C*).

Mutation of Kcnk3 histidine 98 (P1H) to asparagine (P1N) markedly diminishes pH sensitivity of Kcnk3 so that little block is now seen at neutral pH (Fig. 1*D*). Moreover, wild type channels show anomalously low outward current in  $5$  mM potassium compared with  $100$  mM at pH  $7.4$  because of increased proton blockade (Fig. 1*B*) (9) whereas this effect is absent in the mutant (Fig. 1*E*). Otherwise, the P1N mutation did not appear to significantly change channel-gating kinetics, rectification properties, or ion selectivity (Fig. 1, *D* and *E*, and data not shown). These findings suggested that pH inhibition of Kcnk3 resulted from protonation of histidine 98 in the selectivity filter.

**Potassium and Voltage Affect Proton Block**—The influence of external potassium concentration on inhibition of Kcnk3 by proton was next studied (Fig. 2). At  $-120$  mV, with  $5$  mM potassium, Kcnk3 current was half-maximal at pH  $7.6 \pm 0.6$  ( $n = 16$  cells) whereas with  $20$  mM and  $100$  mM potassium, half-block required lowering the pH to  $6.83 \pm 0.01$  and  $6.35 \pm 0.02$ , respectively ( $n = 10$  and  $20$  cells). Moreover, the current-pH relationship with  $5$  mM potassium showed a Hill coefficient of  $0.96 \pm 0.06$  whereas with  $20$  and  $100$  mM potassium, Hill coefficients of  $2.02 \pm 0.14$  and  $1.86 \pm 0.21$  were determined. This suggested that protonation of a single pore site was sufficient to inhibit at low bath potassium whereas protonation



**FIG. 3. Barium block is pH-sensitive in wild type but not mutant Kcnk3 channels.** A, Kcnk3 channels are inhibited by barium in a pH-dependent manner for wild type (P1H) but not for mutant (P1N) channels. Data is shown for inhibition by 1 mM barium at  $-120$  mV in 20 mM potassium ( $n = 7$  cells). B, left, barium off rate ( $\tau_{off}$ ) is pH-dependent for wild type (P1H) but not for mutant (P1N) channels. Oocytes were held at  $-150$  mV for 1 s and then pulsed to 60 mV. Data is shown for inhibition by 1 mM barium with 20 mM potassium ( $n = 7$  cells) as in B. The relaxation was fit with a single exponential. Right, representative trace at pH 6.5 for cells expressing wild type (P1H) or mutant (P1N) channels. Current was normalized to peak. Current relaxation in the absence of barium for P1H was complete in less than 5 ms.

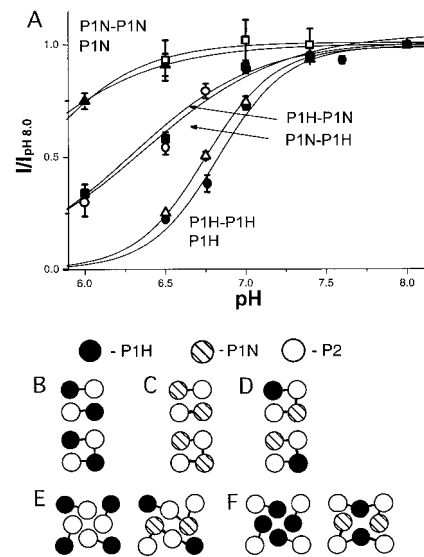
of more than one site was required at higher potassium levels.

Consistent with the idea that proton enters the superficial portion of the Kcnk3 pore to block, inhibition was sensitive to transmembrane voltage. In the presence of 20 mM potassium the apparent fraction of the electric field traversed by proton to reach its site ( $\delta$ ) was estimated to be  $0.08 \pm 0.01$  ( $n = 6$ ; see “Materials and Methods”).

**pH Alters Pore Block by Barium**—To assess the effect of proton blockade on ions entering and leaving the Kcnk3 pore, blockade by external barium was studied. Inward Kcnk3 currents are inhibited by barium in a voltage-dependent manner ( $\alpha\delta = 0.45 \pm 0.01$ ;  $n = 5$  cells) (9), apparently via occupancy of a potassium site in the pore as in 1-P-domain (19, 20) and another 2-P-domain channel (7). Thus, with 20 mM potassium at pH 8.0, Kcnk3 was half-blocked by  $0.40 \pm 0.02$  mM barium with a Hill coefficient of  $1.1 \pm 0.1$  ( $-120$  mV;  $n = 7$  cells). When the pH value was lowered from 8.0 to 6.5, inhibition by 1 mM barium decreased from  $66 \pm 3\%$  to  $37 \pm 4\%$  ( $n = 8$  cells; see Fig. 3A). Although the onset of barium block was not resolved from the capacitance transient associated with a voltage step to  $-150$  mV, release from steady-state barium inhibition at  $-150$  mV could be assessed by a step to 60 mV;  $\tau_{off}$  was slowed  $\sim 2$ -fold (from  $53 \pm 8$  and  $100 \pm 10$  ms) when the pH value was lowered from 8.0 to 6.5 ( $n = 7$  cells) (Fig. 3B).

Studies with the proton-insensitive Kcnk3 channel mutant (P1N) supported the conclusion that the proton(s) that modify barium blockade reside on histidine 98. Thus, P1N channels were inhibited by barium like wild type ( $K_{1/2}$  was  $0.42 \pm 0.06$  with a Hill coefficient of  $0.9 \pm 0.1$ ;  $n = 8$  cells). However, blockade of P1N channels by 1 mM barium was insensitive to pH, showing  $77 \pm 2\%$  and  $75 \pm 3\%$  inhibition at pH 8.0 and 6.5, respectively ( $n = 5$  cells; see Fig. 3A). Moreover, unblock kinetics for P1N channels were less sensitive to pH than wild type channels, showing  $\tau_{off}$  of  $59 \pm 3$  and  $67 \pm 5$  ms at pH 8.0 and 6.5, respectively ( $n = 5$  cells; see Fig. 3B).

**Proton Block of Tandem Dimers**—When two subunits were linked in tandem to produce constructs with four P domains, those with two native subunits (P1H-P1H) or two mutant subunits (P1N-P1N) showed the same sensitivity to proton as channels formed by the related monomer subunits, P1H or P1N (Fig. 4A). Thus, with 20 mM potassium at  $-120$  mV the  $K_{1/2}$  and Hill coefficient for wild type tandems was  $6.75 \pm 0.04$  and  $1.84 \pm 0.11$ , respectively, similar to wild type monomer, suggesting protonation at more than one site. In contrast, tandem constructs with a single histidine in the first or second P1 position (P1H-P1N and P1N-P1H, respectively) showed decreased pH sensitivity and evidence for a single proton blocking



**FIG. 4. Two P1 domains are present in the Kcnk3 pore.** A, number of histidine residues present at the pore determines pH dependence of Kcnk3 channels. The dependence of Kcnk3 currents at  $-120$  mV on bath pH (mean  $\pm$  S.E.) for groups of at least five oocytes in 20 mM potassium solution is shown. The solid line represents a fit of the data in Equation 1.  $IC_{50}$  and the native (P1H-P2-P1H-P2) and mutant (P1N-P2-P1N-P2) tandems of the Hill coefficient were  $6.75 \pm 0.04$  and  $1.84 \pm 0.11$  and  $5.70 \pm 0.15$  and  $0.95 \pm 0.40$ , respectively, whereas the values for mixed tandems were  $6.28 \pm 0.05$  and  $1.10 \pm 0.14$  and  $6.34 \pm 0.04$  and  $1.18 \pm 0.21$  with P1H in the first (P1H-P2-P1N-P2) and downstream (P1N-P2-P1H-P2) position, respectively. B–F, possible arrangement of Kcnk3 subunits assuming the pore is formed by four P domains. B, upper, two P1H subunits; lower, one P1H-P1H tandem in an equivalent configuration. C, upper, two P1N subunits; lower, one P1N-P1N tandem in an equivalent configuration. D, upper, P1H-P1N tandem as in B and C; lower, P1N-P1H tandem. E, left, four P1H subunits arranged as if only P2 domains are pore-forming; right, two P1H-P1N tandem subunits arranged as if some P1 domains are not pore-associated. F, left, four P1H subunits arranged as if only P1 domains are pore-forming; right, two P1N-P1H tandem subunits arranged as if only P1 domains are pore-forming.

site. Values for P1H-P1N channels were  $6.28 \pm 0.05$  and  $1.10 \pm 0.14$  whereas those for P1N-P1H channels were  $6.34 \pm 0.04$  and  $1.18 \pm 0.21$ . These results support the conclusion that two P1H sites are protonated in channels formed by wild type subunits, and one P1H is protonated in mixed tandems whether it resides in the first or downstream position.

## DISCUSSION

**The First P Domain of Kcnk3 Contributes to the Pore**—Here, we demonstrate that Kcnk3 senses pH value changes in the physiological range via protonation of histidine 98 leading to channel blockade. We employ proton blockade to demonstrate that the first P domain in Kcnk3 subunits contributes to pore function and then provide evidence that the pore contains two P1 domains. Four experiments support the conclusions that inhibition results from protonation of sites in the pore and that P1 domains are pore-forming. First, block is mediated by histidine 98, which is located in P1 adjacent to residues that coordinate potassium ions in the ion-conduction pathway (21). Second, block depends on voltage as if the protons enter the transmembrane electric field to bind. Third, protons alter steady-state affinity of barium on its pore site (as well as barium block kinetics). Fourth, increasing external potassium decreases the sensitivity of the channel to proton (perhaps because of increased occupancy of the pore by potassium). Finally, evaluation of linked subunits bearing four P domains (P1-P2-P1-P2) demonstrate that P1 mediates proton blockade in the first or third position arguing for their functional equivalence.



**Kcnk3 Subunit Number**—Because four P domains are necessary and sufficient to form the pore in voltage-gated and inwardly rectifying potassium channels we expect the same number of P domains in each Kcnk3 pore. The arrangement and number of Kcnk3 subunits should account for the following four findings in this report: first, wild type channels are expected to have more than one proton binding site (based on a Hill coefficient of  $\sim 2$  in 20 mM potassium); second, occupancy of one of the sites is sufficient to block (based on a Hill coefficient of  $\sim 1$  in 5 mM potassium); third, the site is formed with histidine residues on the external side of the pore (as the P1N mutant is insensitive); and fourth, only one site is available in channels formed by mixed tandem constructs with a single P1H domain (based on a Hill coefficient of  $\sim 1$  in 20 mM potassium).

The most likely model for pore formation is a dimer with two P1 and two P2 domain as this provides two histidine residues to bind protons in P1H (Fig. 4B, upper) and P1H-P1H channels (Fig. 4B, lower), no histidine sites in P1N (Fig. 4C, upper) and P1N-P1N channels (Fig. 4C, lower), and one histidine in P1H-P1N (Fig. 4D, upper) and P1N-P1H channels (Fig. 4D, lower). It is appropriate to rule out both channels formed by four P2 domains, as P1 domains contribute to pore function (Fig. 4E, left), and channels formed only by the central P domains in linked tandems, because P1H-P1N channels (Fig. 4E, right) show proton blockade. Finally, it seems extremely unlikely but remains possible that four P1 domains form the pore (Fig. 4F) if KCNK P2 domains are non-functional (more on this below). Indeed, four glutamate residues (on four 1P/6TM subunits) form only two proton binding sites in a cyclic nucleotide-gated channel pore (22), and a single proton site is formed by a cluster of four glutamate residues in an L-type cardiac voltage-dependent calcium channel (23). However, to account for our results with mixed tandem constructs, P1 domains would either need to function non-equivalently (which is not what we observed), and/or single protons must block via imidazole/imidazolate pairs (an arrangement that has not been described in other proteins), and/or single protons must block across the mouth of the pore (Fig. 4E, right). Thus, it is reasonable to argue that Kcnk3 channels are homodimeric (Fig. 4B, upper).

Functional evidence that 2-P-domain channels are dimeric has been expected since the isolation of the first channel clones (1). Indeed, an early report argued that a cysteine in the first external loop of KCNK1 was required for both dimer formation and function (24). However, that channel (TWIK1) has subsequently been demonstrated to be non-functional in oocyte plasma membranes, and the cysteine residue revealed to be absent from other KCNK channels (1).

**How Protons Block**—Protonation of Kcnk3 histidine 98 is likely to alter channel gating rather than permeation. Thus, Kim *et al.* (15, 17) found that proton blockade decreased open probability of Kcnk3 and KCNK9 whereas single channel conductance was not significantly altered. Moreover, increasing external permeant ion concentration destabilizes the long-lasting closed state of single KCNK0 channels<sup>2</sup> whereas changing the external permeant ion from potassium to rubidium increases the open probability of single KCNK2 channels (8). These findings suggest that protonation favors Kcnk3 channel closure whereas permeant ions in the pore favor the open state.

The position homologous to P1H in Kcnk3 is also a histidine in KCNK1, -3, -7/8, and -9 but is occupied by aspartate (GYGD) in TOK1 and asparagine (GYGN) in KCNK0, -2, -4, -5, -6, -12, -13, and -15. This indicates that blockade of KCNK5 and -15 channels by external proton proceeds by a different mechanism

than for Kcnk3 and KCNK9. The analogous position in the second P domain of all KCNK channels is aspartate (GYGD) as found in voltage-gated potassium channels; when this P2D was mutated to histidine there was no evidence for Kcnk3 channel function whether a histidine, asparagine, or aspartate was in position 98 (not shown) demonstrating that homologous P1 and P2 residues are not functionally equivalent.

Kir2.1 inward rectifier potassium channels are formed by 1P/2TM subunits and have an arginine residue in the P domain (GYGFR) that has been argued to form an electrostatic barrier to divalent cation permeation, because a histidine at the site (GYGFH) yields channels with increased sensitivity to magnesium until proton levels increase and the site is thought to bear a proton (26); a similar mechanism may explain the effects of pH on barium blockade via P1H in Kcnk3 (Fig. 3).

**Little Is Known about P2 Domains**—Remarkably, a P2 domain has yet to be shown to contribute to pore formation. Thus far, it has been demonstrated that mutations in the TOK1 P2 domain (and flanking transmembrane segments) alter potassium-sensitive gating kinetics without affecting its single channel current (25, 27, 28). Thus, external potassium concentration influences both the magnitude and rate of TOK1 current development, and mutations in some P2 sites modify this effect. As homologous sites in Shaker channels are known to affect the potassium sensitivity of C-type inactivation, these P2 residues have been suggested to contribute to channel regulation. A homodimeric stoichiometry for Kcnk3 demands that P2 domains are, indeed, pore-forming.

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